

Supplementary Information for:

SKA-31, a pharmacological activator of Ca²⁺-activated K⁺ channels, improves cardiovascular function in aging

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Supplementary Table 1 – Details of primary antibodies used for western blotting.

Antibody target	Company/Source	Cat. #/Citation	Dilution
KCa3.1 channel	Santa Cruz Biotechnology	sc-365265	1:1000
KCa2.3 channel	Alomone Labs	APC-025	1:500
SERCA2	Investigator-generated	Chandrasekera and Lytton, 2003, PMID: 12540840	1:1000
Type 1 IP3R	Investigator-generated	Yule et al, 1997, PMID: 9083036	1:1000
eNOS (NOS3)	BD Biosciences	610297	1:1000
PKG1 α	Enzo Life Sciences	ADI-KAP-PK005	1:1000
KCa1.1 channel	Millipore	AB5228	1:500
NFATC1	Santa Cruz Biotechnology	sc-7294	1:1000
Calcineurin	Santa Cruz Biotechnology	sc-17808	1:1000
STIM1	Santa Cruz Biotechnology	sc- 66173	1:1000
ORAI1	Santa Cruz Biotechnology	sc-377281	1:1000
TRPV4 channel	Alomone Labs	ACC-034	1:1000
β -actin	Sigma Aldrich	A1978	1:20,000

Supplementary Table 2 – Details of fluorescently-labelled antibodies used for FACS analysis of isolated splenocytes.

Antibody	Clone	Source	Catalogue #
anti-rat CD8	OX-8	BD Bioscience	558824
anti-rat CD1d	WTH2	BD Biosciences	564834
anti-rat APC-Cy7	OX-1	BD Biosciences	561586
anti-rat CD45RA	OX-33	BD Bioscience	561624
anti-rat CD3	1F4	BD Biosciences	563949
anti-rat CD4	OX-35	BD Biosciences	740138
anti-rat CD25	OX-39	BD Bioscience	565608
anti-rat CD161	10/78	BD Biosciences	561781
anti-rat CD32	D34-485	BD Biosciences	550271
anti-rat FOXP3	FJK16S	eBioscience	17-57773-82

Supplementary Table 3: DNA primers utilized for quantitative PCR measurements.

Primer Target	Sequences
ORAI-1	For: 5'- GTC TCC AGC TGC ACC TC -3' Rev: 5'- TCA CAT CCA ACC ACC ACT C -3'
STIM-1	For: 5'- CAG CCA CTG TAT CAC CTC ATC -3' Rev: 5'- GAC CTC AAT TAC CAT GAC CCT A -3'
IP3R-1	For: 5'- CAC CTA TGA CCA CAC TGT CTC -3' Rev: 5'- AAG AAC GCC ATG AGA GTG AC -3'
KCa3.1 (KCNN4)	For: 5'- CCA CCA CAG CCA CTA GTA GA -3' Rev: 5'- CCA TCA CGT TCC TGA CCA TT -3'
KCa2.3 (KCNN3)	For: 5'- TCA TGG GAA CCT GAA CAC TAA G -3' Rev: 5'- CAT TCC CTG TGC ATC TGA AAG -3'
eNOS	For: 5'- GAC CCT CAC CGA TAC AAC ATA C -3' Rev: 5'- CAG CCA CGT TAA TTT CCA CTG -3'
TRPV4	For: 5'- CCC CGT GGT CTT CAT TCT-3' Rev: 5'- CAT CTG TGC CTG AGT TCT TGT -3'
Phospholipase- γ	For: 5'- ACT TGG CTC ATG GAA GAT ACG -3' Rev: 5'- GGT CCT TGG CTG ATA TAC GAT-3'
NFATC1	For: 5'- TCC GCA ACC AGA GAA TAA CC -3' Rev: 5'- AAC ATT GGC AGG AAG GTA CG -3'
SERCA-2 (ATP2A2)	For: 5'- CTC CGT GTC GAA TAC ATT CAT CT -3' Rev: 5'- TGT GCT CTG TGT AAT GAC TCT G -3'
Kv1.3 (KCNA3)	For: 5'- CCC AGT AAA GCC ACC TTC TC -3' Rev: 5'- GCT CAG TGC CCA GAG TAA TAA A -3'
Calcineurin (PPP3CC)	For: 5'- TCA CCT AGT AAT ACT CGC TAC CT -3' Rev: 5'- CTC CAC AGA TAC AGC ACA CA -3'
KCa1.1 (KCNMA1)	For: 5'- TCT TTC CAG TGC CTT CGT G -3' Rev: 5'- TGG CAG ACT TGT ACT CAA TGG -3'
GAPDH	For: 5'- GTA ACC AGG CGT CCG ATA C -3' Rev: 5'- TCT CTG CTC CTC CCT GTT C -3'
β -actin (ACTb)	For: 5'- GGA TGT CAA CGT CAC ACT TCA -3' Rev: 5'- CAG GTC ATC ACT ATC GGC AA -3'

Supplementary Table 4- Functional and structural parameters measured following 8 weeks of treatment with either vehicle or SKA-31 (10 mg/kg daily) in young and aged rats. Data are presented as means \pm SD calculated from 5-6 animals in each treatment group. The asterisk (*) indicates a statistically significance between the aged rats treated with either vehicle or SKA-31 and the young vehicle treated group; $P < 0.05$. The # sign signifies a statistical difference between aged SKA-31 treated rats vs. the vehicle treated aged animals ($P < 0.05$), as determined by one way ANOVA and a Tukey post hoc test.

CA-Chamber Area; **d**-diastole; **s**-systole; **EDV**- End Diastolic Volume; **ESV**-End Systolic Volume; **EF**: Ejection Fraction; **SV**: Stroke Volume; **IVS**-Interventricular Septum; **LVPW**-Left Ventricular Posterior Wall; **LVID**- Left Ventricular Internal Diameter; **FS**: Fractional Shortening

Parameters measured	Young vehicle treated n=5	Aged vehicle treated n=6	Aged SKA-31 treated n=6
4CA _d (mm ²)	84.01 \pm 5.74	96.59 \pm 2.68*	109.24 \pm 4.27*#
4CA _s (cm ²)	39.92 \pm 1.32	49.28 \pm 2.52*	49.03 \pm 3.92*
2CA _d (mm ²)	87.15 \pm 7.73	97.02 \pm 3.13*	105.40 \pm 3.97*
2CA _s (mm ²)	43.81 \pm 5.66	49.04 \pm 2.37	51.65 \pm 4.03*
EDV (cm ³)	0.57 \pm 0.06	0.56 \pm 0.08	0.65 \pm 0.07*#
ESV (cm ³)	0.18 \pm 0.01	0.20 \pm 0.02	0.21 \pm 0.03
EF (%)	68.44 \pm 2.29	63.41 \pm 1.57*	67.83 \pm 1.77#
SV (cm ³)	0.39 \pm 0.08	0.36 \pm 0.04	0.44 \pm 0.05#
IVS _d (mm)	1.88 \pm 0.20	2.25 \pm 0.34	2.02 \pm 0.13
IVS _s (mm)	2.72 \pm 0.16	3.01 \pm 0.44	3.20 \pm 0.17*
LVPW _d (mm)	2.64 \pm 0.62	3.22 \pm 0.65*	3.28 \pm 0.73*
LVPW _s (mm)	3.20 \pm 0.37	3.83 \pm 0.35*	3.80 \pm 0.73*
LVID _d (mm)	7.78 \pm 0.97	8.12 \pm 0.51	8.40 \pm 0.49
LVID _s (mm)	4.94 \pm 0.79	5.48 \pm 0.48	5.31 \pm 0.34
FS%	35.36 \pm 2.3	32.30 \pm 1.63*	36.60 \pm 2.14#
Heart rate (bpm)	343 \pm 29	301 \pm 23	291 \pm 19

Supplementary Table 5 – Concentrations of common cytokines and chemokines measured in plasma from young and aged male rats treated daily for 8 weeks with either drug vehicle or SKA-31 (10 mg/kg). Plasma samples were collected at the time of euthanasia, approximately 18 h following the final administration of either vehicle or drug. Values were determined by a fluorescence-based multiplex array, and readings from plasma samples (n = 4-6 animals per group) were compared with a standard curve prepared for each cytokine/chemokine to determine the presented values. Measured concentrations are expressed as mean \pm S.D. and were examined statistically by ANOVA and a Tukey's post-hoc test.

* indicates a statistically significant difference vs. the vehicle-treated young group, $P < 0.05$

indicates a statistically significant difference vs. the vehicle-treated aged group, $P < 0.05$

† indicates that fluorescence-based readings were below that for the lowest point (i.e. 3.6 pg/ml) on the internal standard curve.

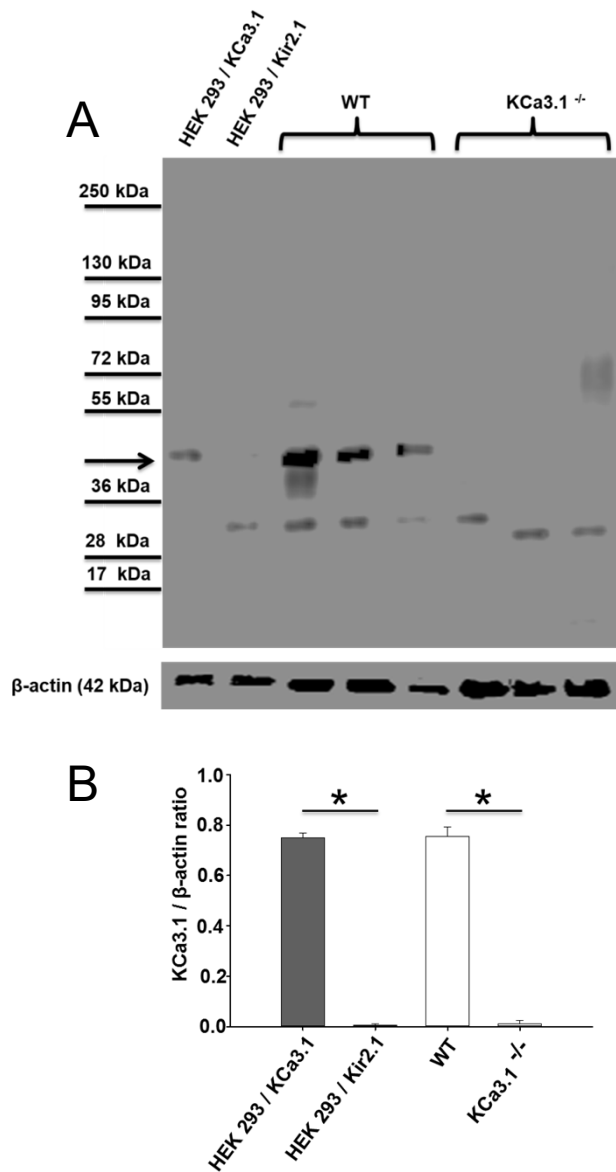
Supplementary Table 5, continued

	Young + Vehicle	Young + SKA-31	Aged + Vehicle	Aged + SKA-31
Cytokine/ Chemokine	Plasma Concent. (pg/ml)	Plasma Concent. (pg/ml)	Plasma Concent. (pg/ml)	Plasma Concent. (pg/ml)
Eotaxin	5.3 ± 2.7	7.1 ± 2.9	12.1 ± 3.7*	14.2 ± 2.1*
EGF	< 0.05†	< 0.05†	0.9 ± 0.8	0.9 ± 1.1
Fractalkine	40.8 ± 20.1	48.5 ± 15.3	61.0 ± 16.9	56.6 ± 17.7
G-CSF	27.7 ± 9.0	15.1 ± 7.9	56.6 ± 29.2	72.9 ± 48.4
GM-CSF	68.1 ± 69.1	96.3 ± 42.5	187.8 ± 158.4	411.6 ± 100.1*#
GRO/KC	50.2 ± 19.5	150.4 ± 55.6	136.8 ± 206.3	141.3 ± 158.9
IFN-gamma	< 3.6†	< 3.6†	< 3.6†	< 3.6†
IL-1α	32.1 ± 6.8	31.1 ± 12.6	85.2 ± 40.6	106.3 ± 69.4
IL-1β	160.3 ± 172.4	343.0 ± 462.4	126.6 ± 125.8	128.4 ± 87.9
IL-2	57.4 ± 45.2	46.0 ± 16.9	94.4 ± 44.8	96.8 ± 42.7
IL-4	13.0 ± 10.6	15.4 ± 7.6	32.1 ± 17.9	44.7 ± 12.6*
IL-5	41.6 ± 16.1	62.6 ± 9.7	73.8 ± 25.1*	80.5 ± 10.7*
IL-6	285.4 ± 144.8	444.1 ± 377.7	929.4 ± 527.5*	1278.4 ± 451.1*
IL-10	166.8 ± 181.3	291.7 ± 362.6	124.7 ± 74.9	106.9 ± 73.2
IL-12	82.9 ± 23.8	140.1 ± 27.5	290.4 ± 123.4*	322.4 ± 96.7*
IL-13	7.2 ± 7.8	10.3 ± 10.1	21.9 ± 10.7*	36.3 ± 10.5*
IL-17A	10.4 ± 8.4	6.4 ± 2.4	15.9 ± 10.4	21.5 ± 13.9
IL-18	114.2 ± 77.1	150.5 ± 35.5	303.0 ± 119.9*	236.6 ± 70.7
IP-10	179.8 ± 76.0	357.1 ± 155.5	335.1 ± 97.4*	264.8 ± 78.3
Leptin	26372.1 ± 17368.6	25468.1 ± 14575.2	31137.9 ± 9438.4	26140.9 ± 5462.4
LIX	838.8 ± 496.3	1069.7 ± 365.0	2152.1 ± 967.6	2391.8 ± 1406.8
MCP-1	419.6 ± 266.0	1318.9 ± 894.6	1325.7 ± 697.1*	1221.0 ± 425.2*
MIP-1a	5.5 ± 1.4	8.8 ± 3.2	10.4 ± 4.1	14.1 ± 7.7*
MIP-2	137.3 ± 41.1	88.5 ± 40.4	181.2 ± 36.5	200.5 ± 29.7*
RANTES	299.2 ± 181.0	522.9 ± 374.7	1076.3 ± 660.5	1328.3 ± 1113.2
TGFβ-1	8894.1 ± 9351.4	39599.3 ± 21758.2*	39409.7 ± 23903.8	57946.4 ± 35586.8*
TGFβ-2	1183.3 ± 610.6	4045.3 ± 1748.9*	3175.1 ± 1893.4	3794.4 ± 1956.8*
TGFβ-3	45.0 ± 8.7	62.2 ± 19.2	60.6 ± 22.5	69.0 ± 21.2
TNF-α	11.2 ± 5.1	9.7 ± 2.2	20.8 ± 7.4*	25.2 ± 5.3*
VEGF	3.3 ± 2.0	21.4 ± 22.4	41.2 ± 7.5*	23.0 ± 29.3

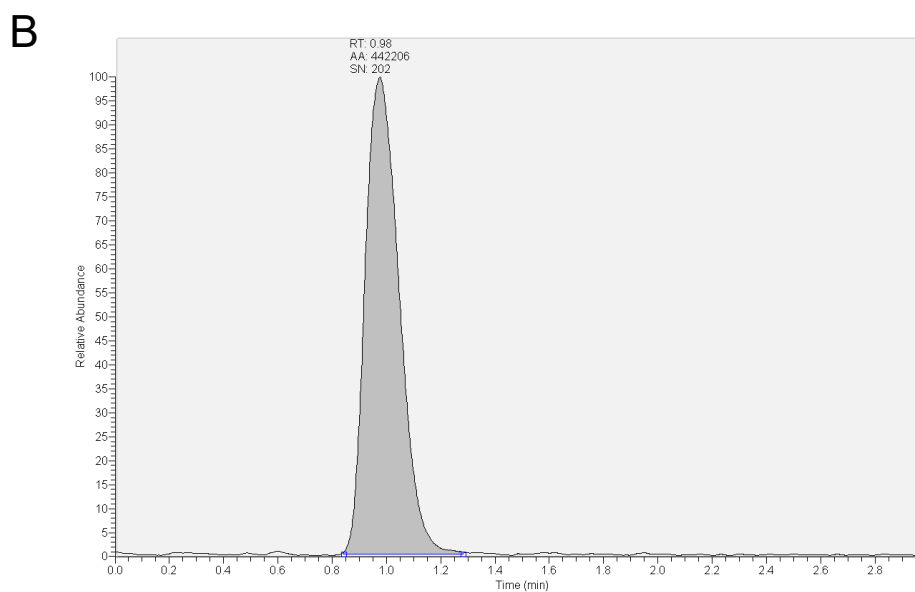
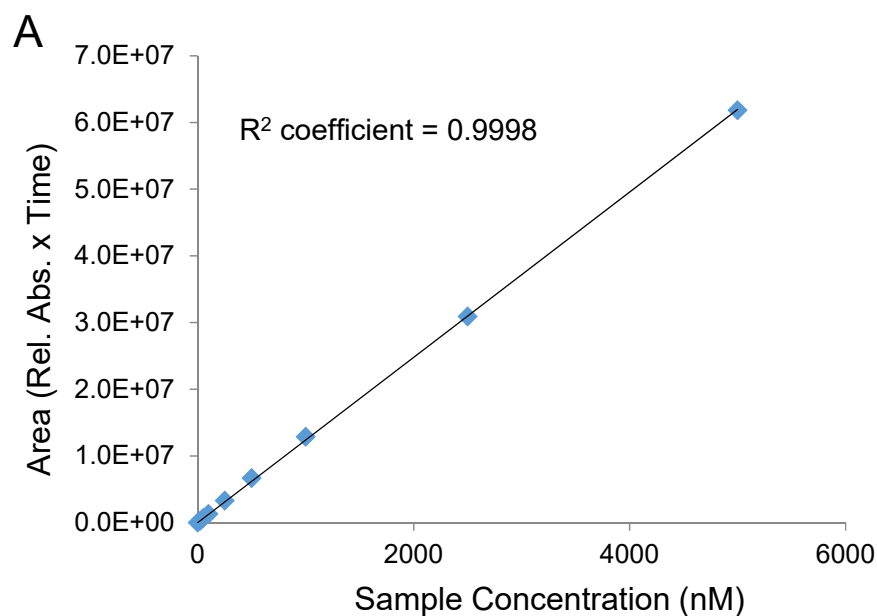
Supplementary Table 6 – Measurements of common chemical and protein constituents in plasma from young and aged male Sprague-Dawley rats treated daily with either vehicle or SKA-31 (10 mg/kg) for 8 weeks. Values are expressed as mean \pm S.D., N = 6-8 for each group. Reference values for Sprague-Dawley rats are expressed as ranges, and are based on routine testing performed by the supplier Charles River (see their website for full description).

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK, creatinine kinase; T. Bili, total bilirubin.

Parameter	Young+Vehicle	Young+SKA-31 (10 mg/kg)	Aged+Vehicle	Aged+SKA-31 (10 mg/kg)	Reference Values (17 weeks and older)
Glucose (mmol/L)	11.3 \pm 2.2	10 \pm 1.2	11.2 \pm 0.4	12.9 \pm 1.3	6.88-10.21
Urea (BUN) (mmol/L)	6.9 \pm 1.1	6.8 \pm 0.6	7.3 \pm 0.7	7.2 \pm 0.2	3.81-7.34
Creatinine (μ mol/L)	27.8 \pm 3.4	24.2 \pm 2.3	33.3 \pm 3.6	33.0 \pm 1.8	26.52-44.21
Phosphorus (mmol/L)	2.5 \pm 0.2	2.4 \pm 0.2	1.8 \pm 0.2	2.1 \pm 0.2	1.18-2.71
Sodium (mmol/L)	134.3 \pm 7.4	139.2 \pm 6.3	138.7 \pm 2.8	140.2 \pm 2.0	137-147
Chloride (mmol/L)	96 \pm 5.6	96.7 \pm 3.6	96.5 \pm 2.4	97.8 \pm 1.3	98-106
Bicarbonate (mmol/L)	22.5 \pm 4.0	22.8 \pm 1.5	21.0 \pm 0.9	19.7 \pm 2.1	18-28
Total Protein (g/L)	60.0 \pm 4.7	56.5 \pm 3.0	57.7 \pm 4.1	55.3 \pm 1.0	56-75
Albumin (g/L)	31.3 \pm 2.0	31.8 \pm 2.0	30.0 \pm 1.7	29.7 \pm 1.2	36-47
Globulin (g/L)	28.7 \pm 3.3	24.7 \pm 1.2	27.7 \pm 2.8	25.7 \pm 0.8	18-29
ALT (IU/L)	79.0 \pm 8.3	48.2 \pm 8.7	90.5 \pm 49.6	91.5 \pm 33.9	19-88
AST (IU/L)	152.7 \pm 29.0	132.8 \pm 43.6	188.5 \pm 51.3	208.2 \pm 31.8	60-217
ALP (IU/L)	89.3 \pm 37.0	118.2 \pm 46.2	46.3 \pm 22.3	28.8 \pm 2.9	26-131
T. Bili (Total) (μ mol/L)	2.4 \pm 0.4	1.6 \pm 0.1	2.5 \pm 0.3	2.5 \pm 0.4	0.68-3.42
CK (IU/L)	615.2 \pm 169.8	627.5 \pm 235.0	694.8 \pm 356.7	651.5 \pm 324.9	460-1230
Testosterone (ng/ml)	1.1 \pm 0.3	1.3 \pm 0.2	1.5 \pm 0.2	1.2 \pm 0.2	3-6 ng/ml

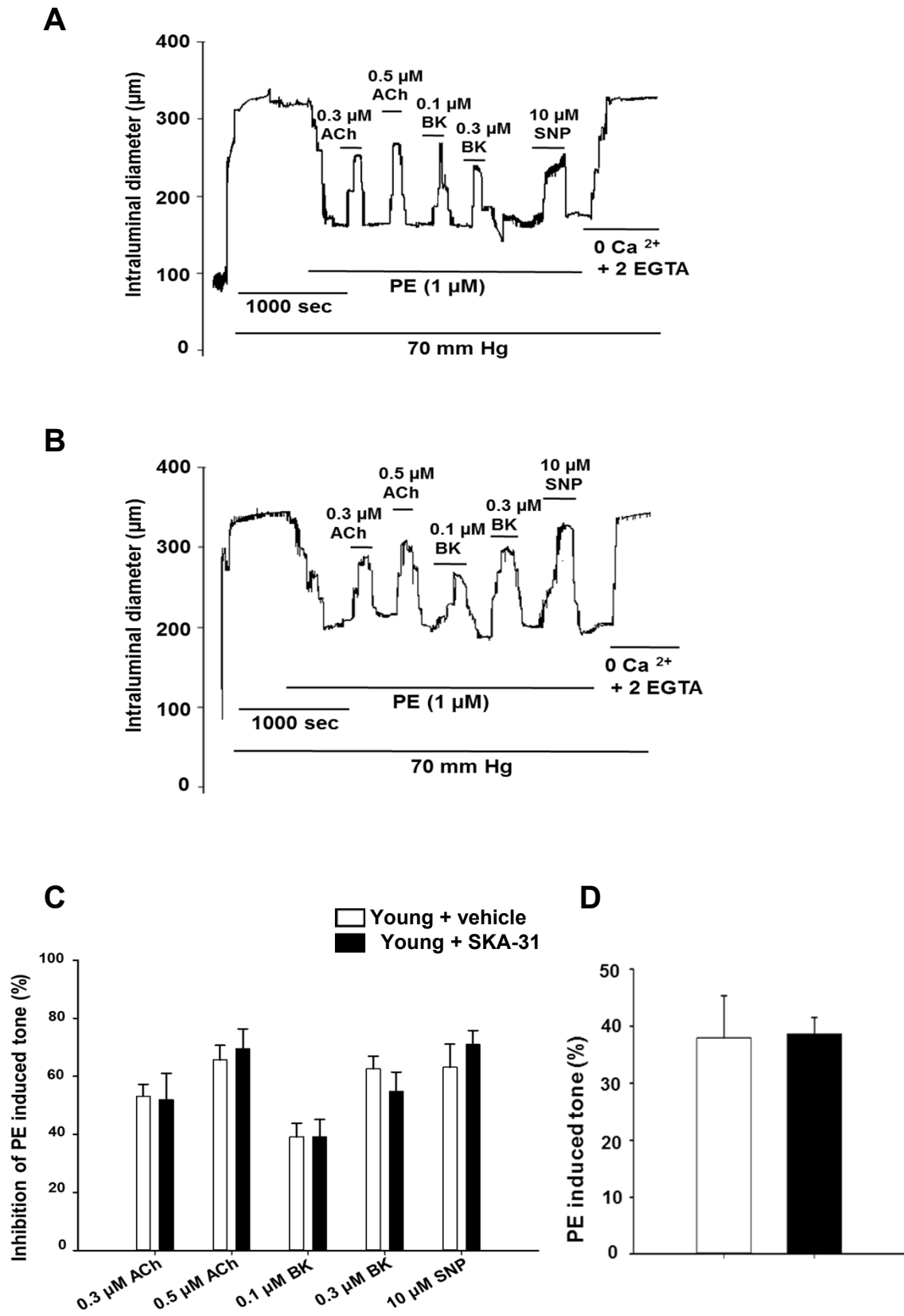


Supplementary Figure 1 - Detection of KCa3.1 protein in mesenteric arteries from WT and KCa3.1^{-/-} mice. (A) Western blot showing immuno-reactive bands detected with an anti-KCa3.1 channel antibody (clone D5, Santa Cruz Biotechnology) in mesenteric arteries from 3 individual wild-type (WT) and KCa3.1 knockout (^{-/-}) mice. HEK 293 cells transfected with cDNA encoding either human KCa3.1 channel or rat Kir2.1 channel were used as positive and negative staining controls, respectively. KCa3.1 channel protein is denoted by the arrow on the left-hand side. Detection of β-actin was used as a loading control. (B) Histogram quantifying the protein expression ratio of KCa3.1 to β-actin in mesenteric artery homogenates from WT and KCa3.1^{-/-} mice, along with lysates from transfected HEK 293 cells. The asterisk signifies a statistical difference ($P < 0.05$) between the indicated groups, as determined by ANOVA and a Tukey post-hoc test, (WT, $n = 4$ and KCa3.1^{-/-}, $n = 6$ animals).



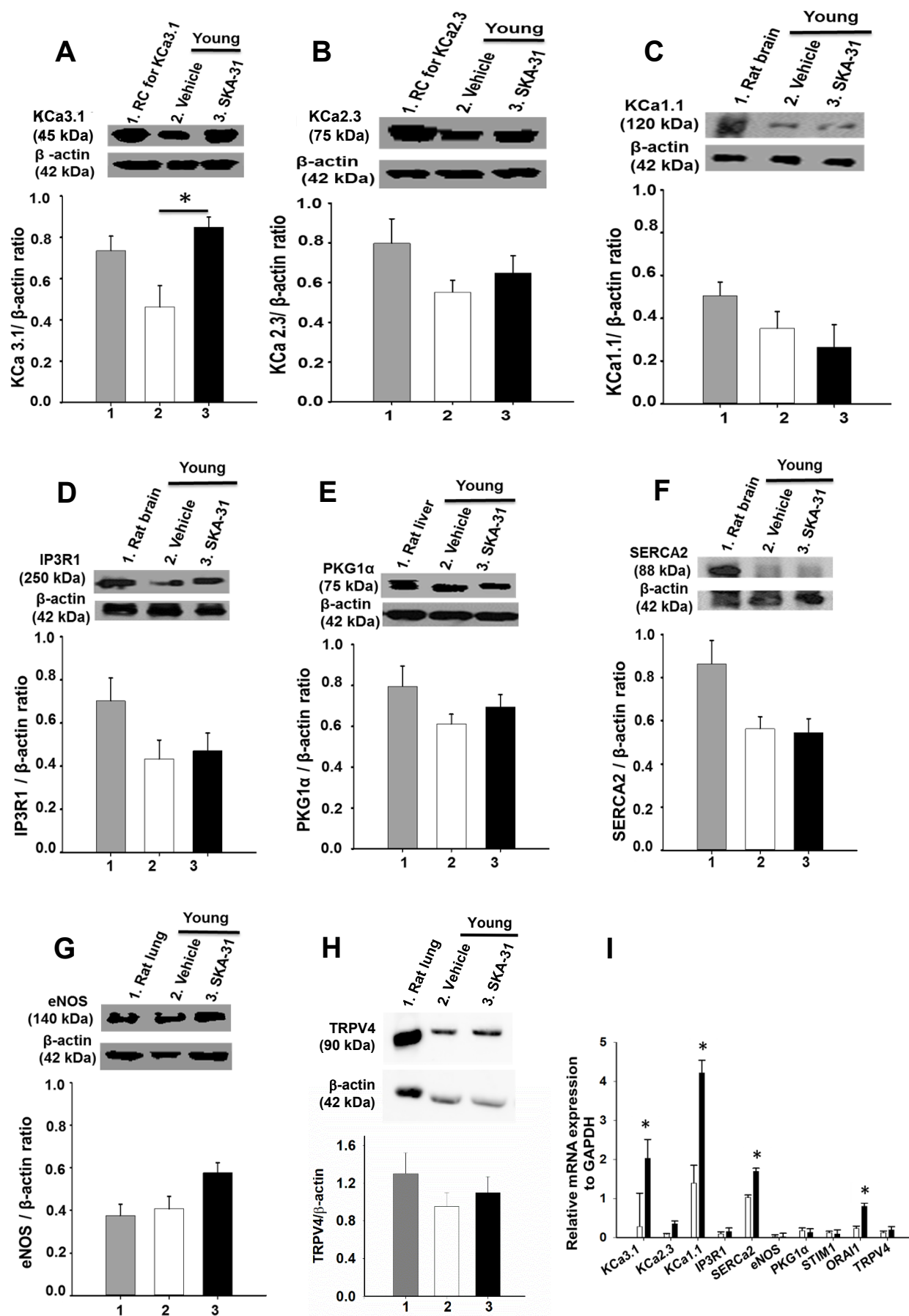
Supplementary Figure 2 – Calibration curve and chromatogram for SKA-31 detection in rat plasma. Panel A displays a calibration curve for SKA-31 concentrations ranging from 25 nM to 5 μ M of SKA-31 analyzed by LC/MS, as described in the Methods and Materials. The representative chromatogram in panel B illustrates the detection of a 50 nM sample of SKA-31.

Supplementary Figure 3 – Long-term SKA-31 administration does not alter vascular reactivity in isolated arteries from young rats. Panels A and B display representative tracings of vasodilatory responses evoked by acetylcholine (ACh, 0.3 and 0.5 μ M), bradykinin (BK, 0.1 and 0.3 μ M) and sodium nitroprusside (10 μ M) in phenylephrine (PE) pre-constricted mesenteric arteries isolated from young male rats treated with either vehicle (A) or 10 mg/kg SKA-31 (B). The horizontal bars above and below the intraluminal diameter tracings indicate bath application of individual agents. The histogram in panel C quantifies the inhibition of PE induced tone in isolated mesenteric arteries from vehicle and SKA-31 treated rats. Panel D displays the degree of PE-induced constriction (i.e. percent reduction in maximal passive intraluminal diameter) in small mesenteric arteries from vehicle and SKA-31 treated rats. Data in panels C and D are displayed as mean \pm S.D and were calculated from 6-8 animals per experimental condition. No statistical differences were observed between the two groups, as determined by an unpaired Student's t-test.



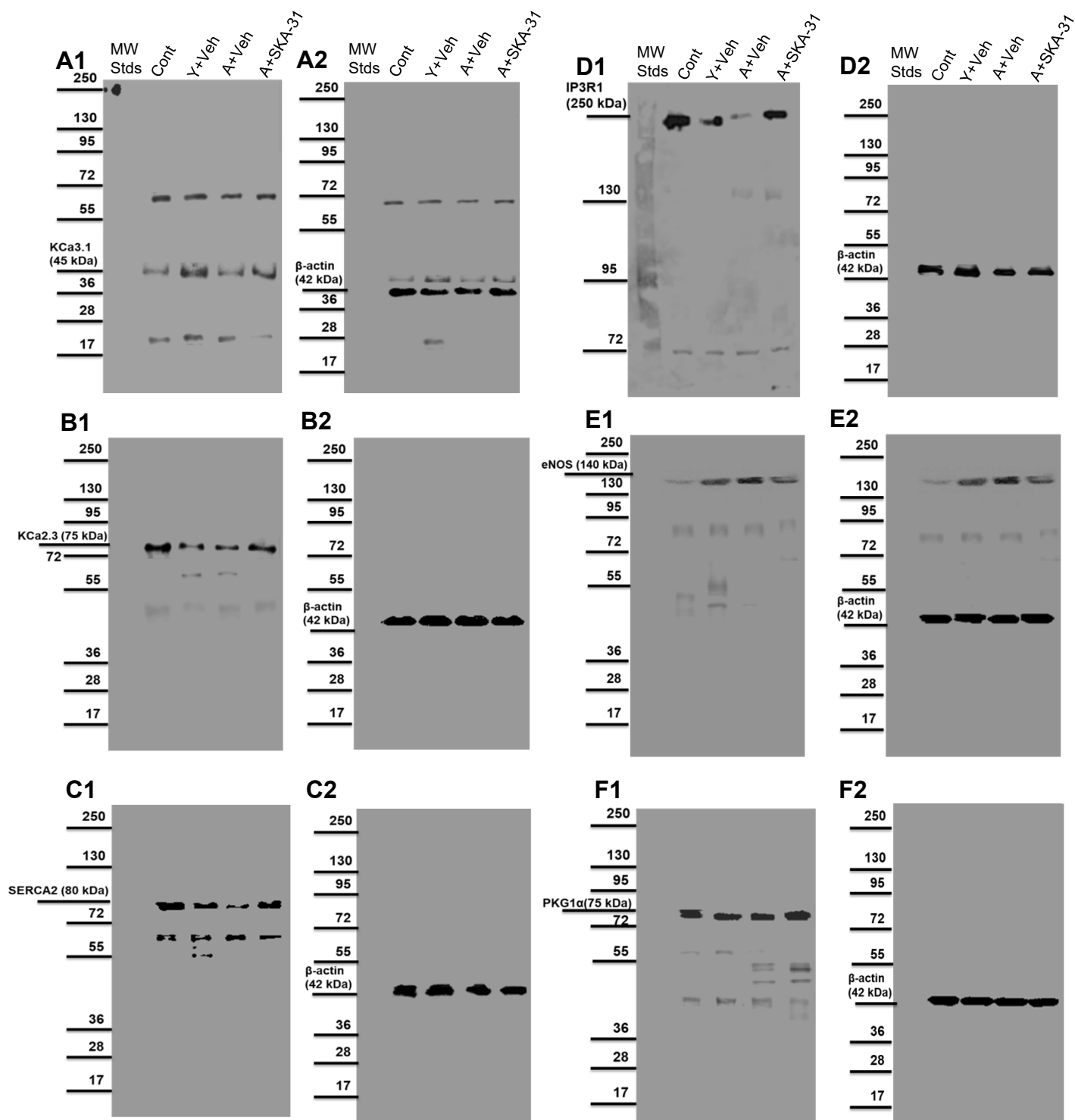
Supplementary Figure 3

Supplementary Figure 4 - SKA-31 administration has minimal effect on the expression of key proteins associated with vasoactive responses in mesenteric arteries from young rats. Panels A-H display representative western blots and quantitative analysis of KCa3.1 channel (A), KCa2.3 channel (B), KCa1.1 channel (C), type 1 IP3 receptor (IP3R1) (D), type 1 α protein kinase G (PKG1 α) (E), SERCA2 ATPase (F), endothelial NO synthase (eNOS) (G) and TRPV4 channel (H) in mesenteric artery homogenates derived from vehicle (white bars) and SKA-31 treated young rats (black bars). For each primary antibody tested, a lysate derived from a tissue or recombinant cell (RC) line expressing the target protein was included as a positive control, and is displayed in lane 1 of each blot. Staining intensities of the selected immuno-reactive bands are expressed as a ratio with detected β -actin expression in the same homogenate. The asterisk signifies a statistical difference between the indicated groups (n = 3-4 animals), as determined by ANOVA and a Tukey post-hoc test, P < 0.05. Panel I shows the mRNA levels of key signalling proteins in mesenteric arteries from vehicle and SKA-31 treated young rats, as determined by qPCR analysis. Data are normalized to the expression level of GAPDH mRNA detected in the same mesenteric arteries from vehicle and SKA-31 treated young rats and was calculated using REST software. Statistical analyses were performed using an unpaired Student's t-test (n = 4-5 animals/group).

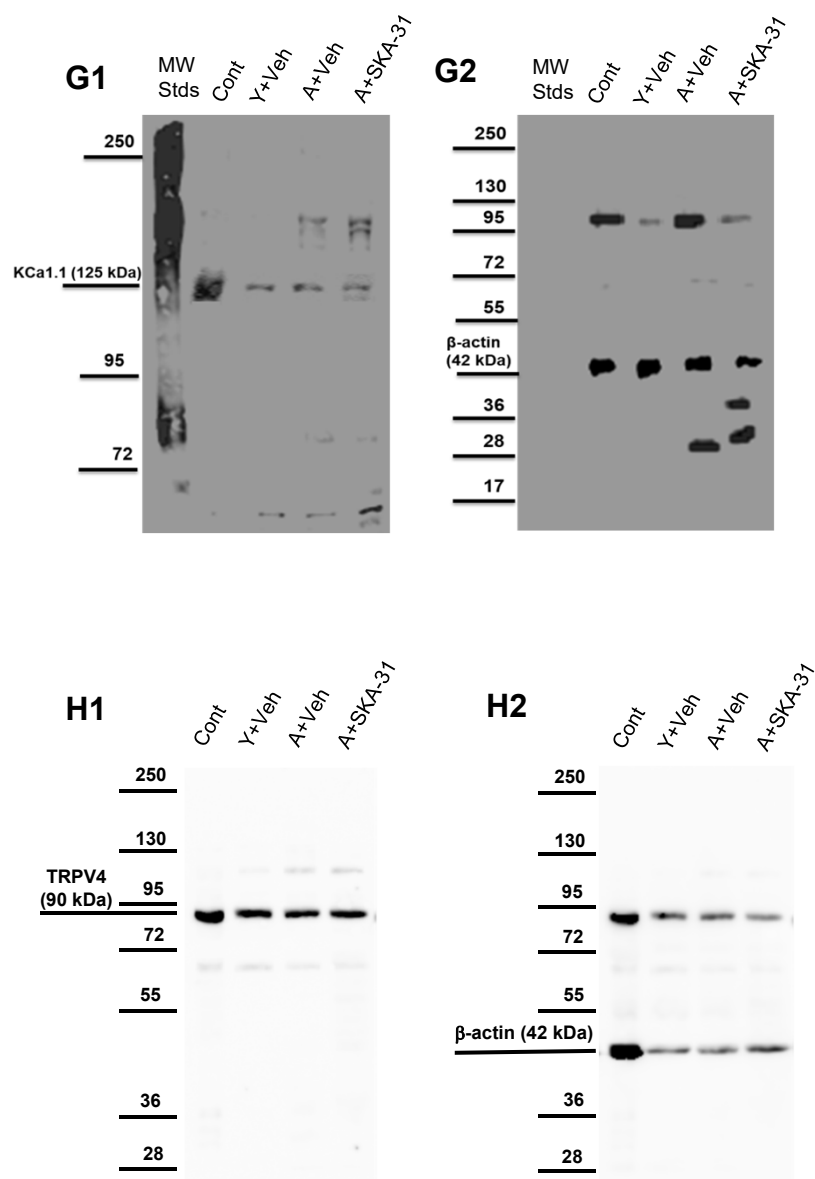


Supplementary Figure 4

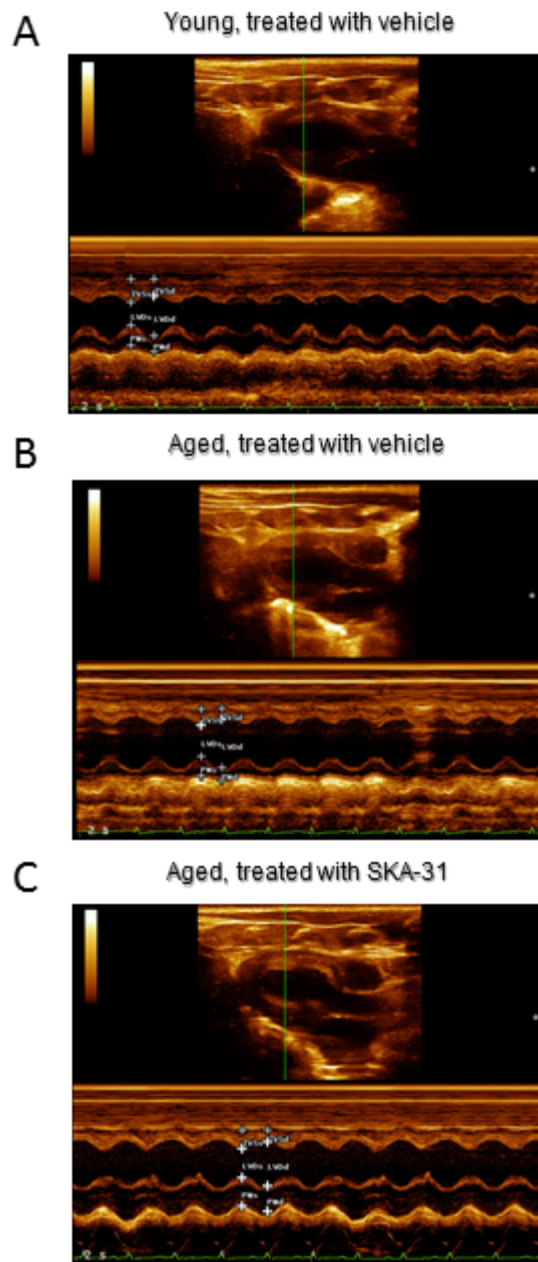
Supplementary Figure 5 – Representative full length western blots for target proteins detected in small mesenteric arteries. Third and fourth order mesenteric arteries were isolated from young rats treated with vehicle (lane denoted Y+Veh), or aged rats administered either vehicle (A+Veh) or 10 mg/kg SKA-31 (A+SKA-31) for 8 weeks. Tissues or cells with expected positive reactivity for the primary antibody in question were utilized as positive controls (lane denoted Cont) for the immuno-detection process. The electrophoretic positions of molecular weight markers are indicated on the left-hand side of each blot. The identification and molecular weight of the target protein of interest for each blot are also indicated on the left-hand side. Blot images are arranged in pairs (e.g. A1 and A2), with the target protein of interest shown in blot #1 and associated β -actin staining on either the same membrane or a parallel membrane displayed in the blot labelled #2. In some cases, the β -actin blot displays protein bands associated with the initial detection of the target protein of interest. This signal carryover occurred mainly from the use of monoclonal antibodies to detect each separate target, and the use of a common HRP-linked secondary antibody. Note that blots were not chemically stripped between the first and second rounds of immuno-detection with primary antibodies.



Supplementary Figure 5

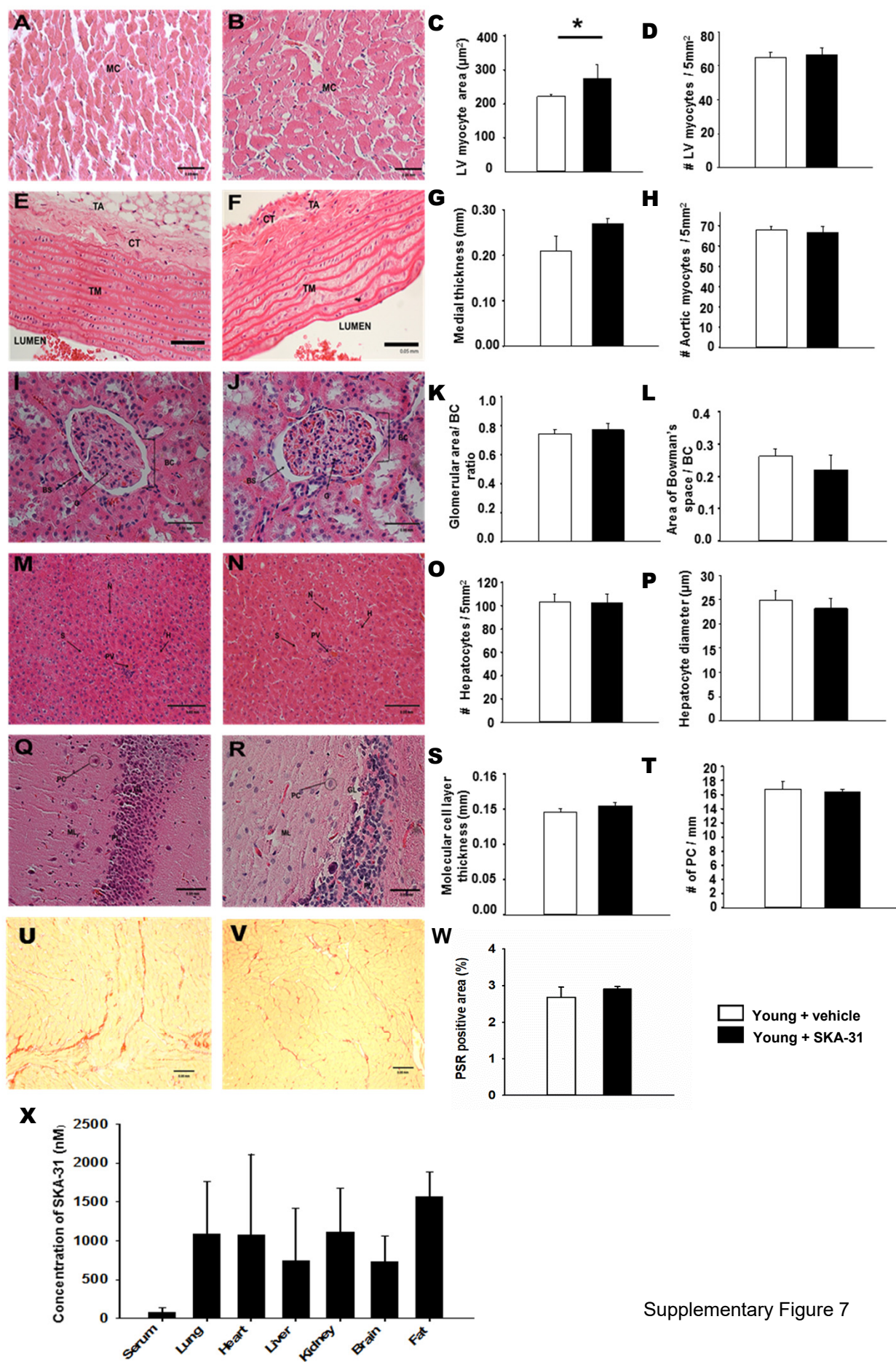


Supplementary Figure 5 (cont'd)

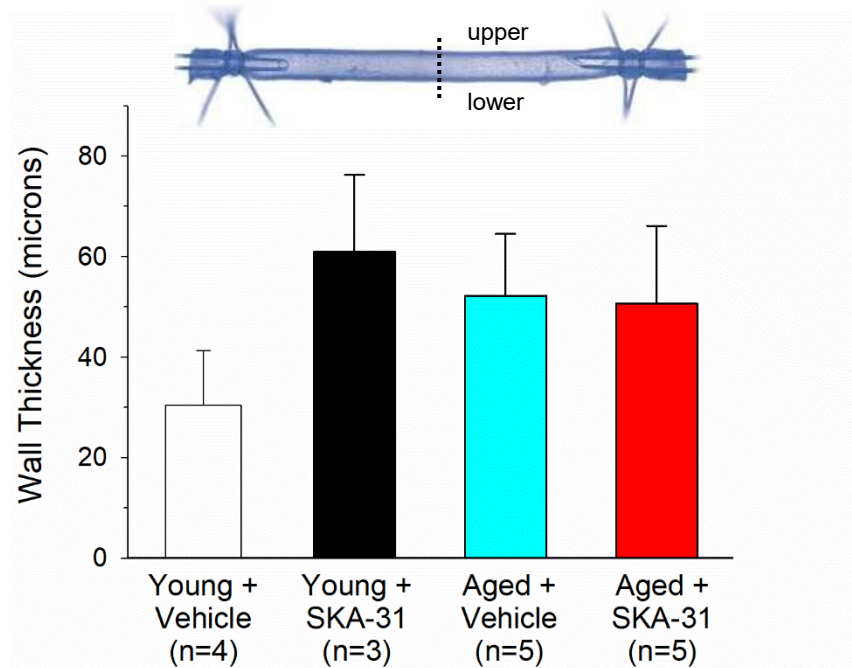


Supplementary Figure 6 - Representative left ventricular M-mode echocardiographic recordings from A) Young rats treated with vehicle, B) Aged rats treated with vehicle, and C) Aged rats treated with 10 mg/kg SKA-31.

Supplementary Figure 7 – Histopathological examination of select tissues from young rats following 8-week administration of either vehicle or SKA-31 (10 mg/kg). H&E staining (400x magnification) of the left ventricular free wall from rats treated with either vehicle (panel A) or SKA-31 (panel B). Note that the scale bar displayed in the bottom right corner of these panels and all subsequent histological images represents 50 microns; MC: myocyte. Panels C and D quantify the size of individual LV myocytes ($n \geq 50$ cells analyzed per tissue) and density of LV myocytes (i.e. cell number per 5mm^2 of tissue area) in each group. The H&E stained sections in panels E-F (400x) display cross sections of thoracic aorta isolated from vehicle (E) and SKA-31 treated rats (F). TA: tunica adventitia, TM: tunica media, CT: connective tissue. Medial layer thickness of the thoracic aorta and the number of aortic myocytes per 5mm^2 of tissue area are shown in panels G and H, respectively. Panels I-J display kidney sections (400x magnification) obtained from vehicle (I) and SKA-31(J) treated rats. BC: Bowman's capsule, BS: Bowman's Space, G: Glomerulus. Histograms in panels K and L quantify the glomerular size relative to the area of Bowman's capsule, and the area of Bowman's space relative to Bowman's capsule, respectively. H&E staining of liver sections (400x) from vehicle (M) and SKA-31(N) treated rats. H: Hepatocyte, N: Nucleus, S: Sinusoid, PV: Portal Vein. Measured hepatocyte density (cells per 5mm^2 of tissue area) and diameter are shown in panels O and P, respectively. Panels Q-R display H&E staining of brain cerebellar sections (400x) from vehicle (Q) and SKA-31 (R) treated animals. GL: Granule cell Layer, PC: Purkinje Cells, ML: Molecular cell Layer. Panels S and T quantify molecular cell layer thickness in the cerebellum (S) and the number of Purkinje cells per millimetre length of the molecular layer (T). Representative Picrosirius red-stained sections of the left ventricular (LV) free wall from vehicle and SKA-31 treated rats are shown in panels U and V, respectively. The histogram in panel W quantifies the Picrosirius red (PSR)-positive area in LV tissue from each group. All structural measurements in stained sections were carried out using ImageJ software. Panel X shows the total plasma and tissue concentrations of SKA-31 in young treated rats, as determined by LC/MS analyses. Tissues and plasma were collected at the time of euthanasia, approximately 18 hours following the final i.p. injection of SKA-31. Adipose (fat) tissue was obtained from the abdominal region. No SKA-31-like chemical signal was detected in material from vehicle treated animals. Statistical analyses were performed using either an unpaired Student's t-test or an ANOVA and Tukey's post-hoc test (panel X only). An asterisk indicates a statistically significant difference between groups, $P < 0.05$ ($n = 5$ animals/group).

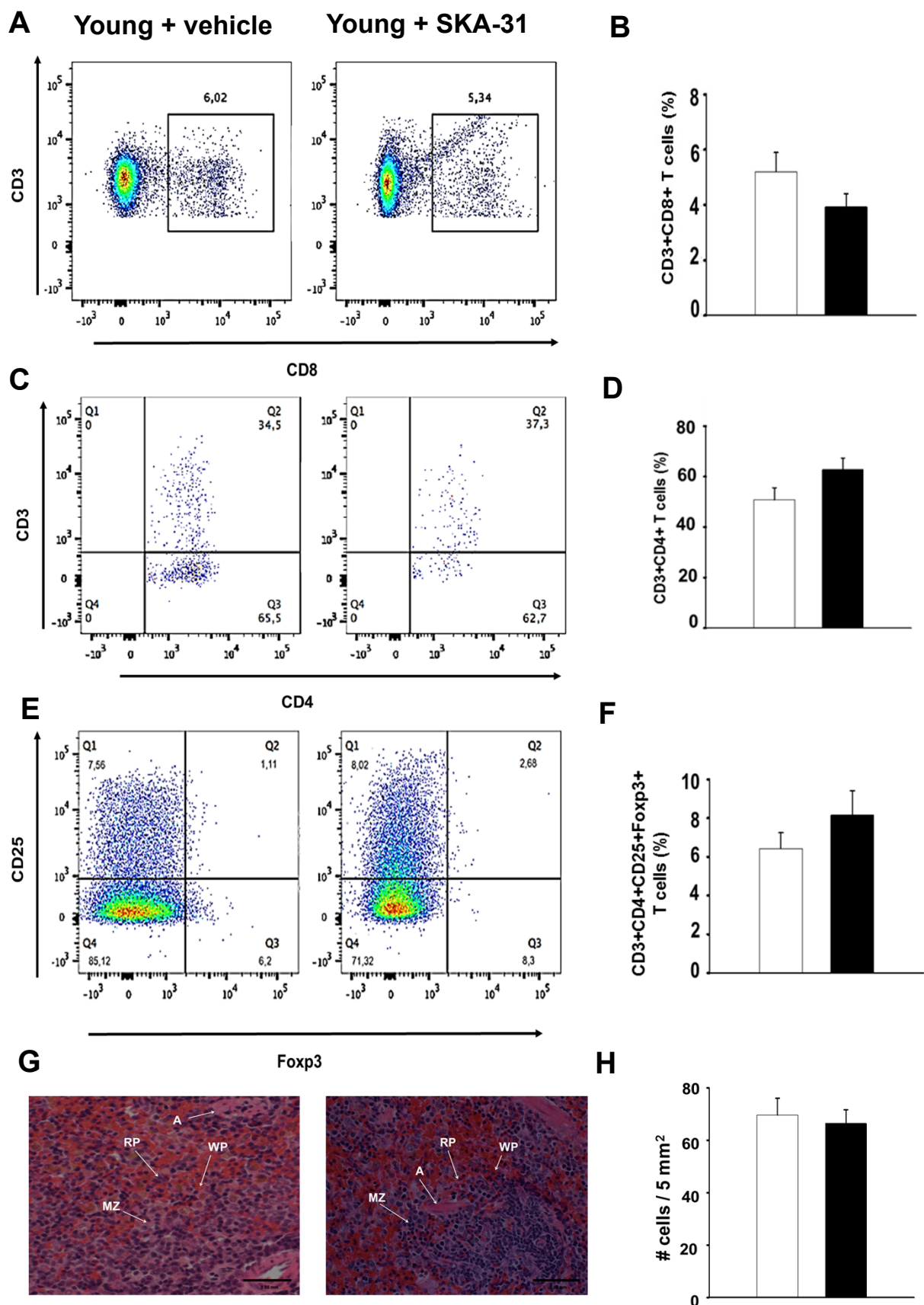


Supplementary Figure 7



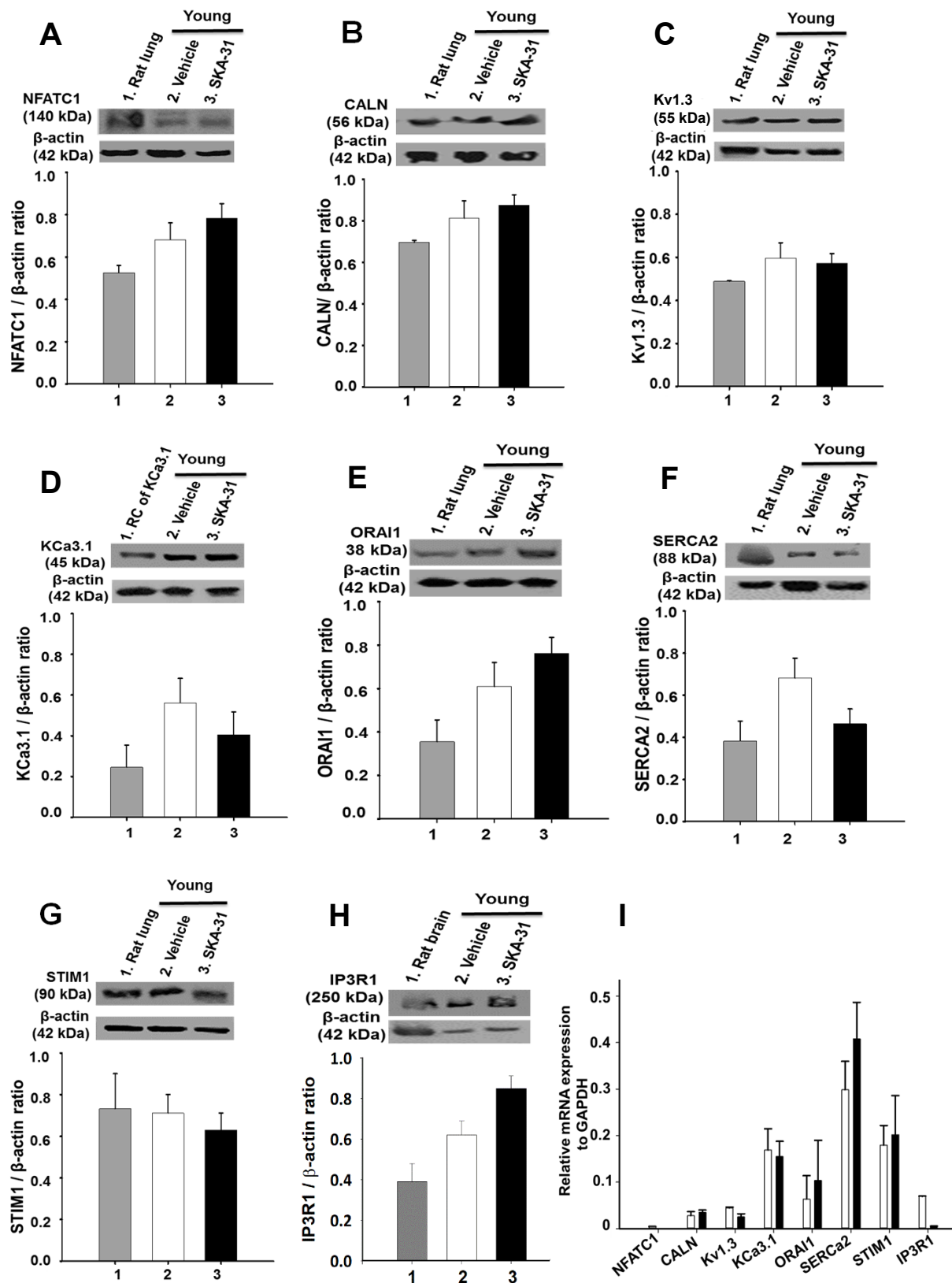
Supplementary Figure 8 – Effects of SKA-31 administration on wall thickness in pressurized mesenteric arteries from young and aged rats. Histogram displays the average wall thickness in pressurized small mesenteric arteries from young and aged rats treated with either vehicle or SKA-31. At the end of the pressure myography protocol, arteries were exposed to Krebs' buffer containing nominally free external calcium and 2 mM EGTA to generate maximal passive arterial diameter. The thickness of each arterial wall (upper and lower, as shown in the cannulated vessel image in the inset) was then measured as the difference between the outer and inner vessel diameter; the dotted line in the image shows the vertical plane used for the measurements. The two values of wall thickness calculated for each artery were then averaged. Data are displayed as means \pm S.D. for each treatment group; N indicates the number of animals. No statistical differences were noted amongst groups, as determined by ANOVA and a Tukey's post-hoc test.

Supplementary Figure 9 – Long-term SKA-31 administration does not promote a pro-inflammatory state. Panels A-F display representative flow cytometry profiles and quantification of immune cell subsets isolated from the spleens of vehicle (n = 5) and SKA-31 treated (n = 6) young rats. Total T cells in the lymphocyte population were gated as CD3⁺ and further gated to CD8⁺ (panels A and B) and CD4⁺ (panels C and D) for helper and cytotoxic T cells, respectively; regulatory T cells were gated with CD4⁺CD25⁺Foxp3 (panels E and F). The two groups were compared statistically using a Mann-Whitney test, and the data are expressed as means \pm S.D. Panel G show H&E staining of spleen sections (400x magnification) from vehicle (left) and SKA-31 treated (right) young animals. The scale bar in the bottom right corner of each image represents 50 microns; RP: Red Pulp, WP: White Pulp, MZ: Marginal Zone, A: Artery. Panel H displays the quantification of cells per 5mm² of H&E stained sections of intact spleen from vehicle (white bar) and SKA-31 treated young rats (black bars). Image analysis was performed using ImageJ software.



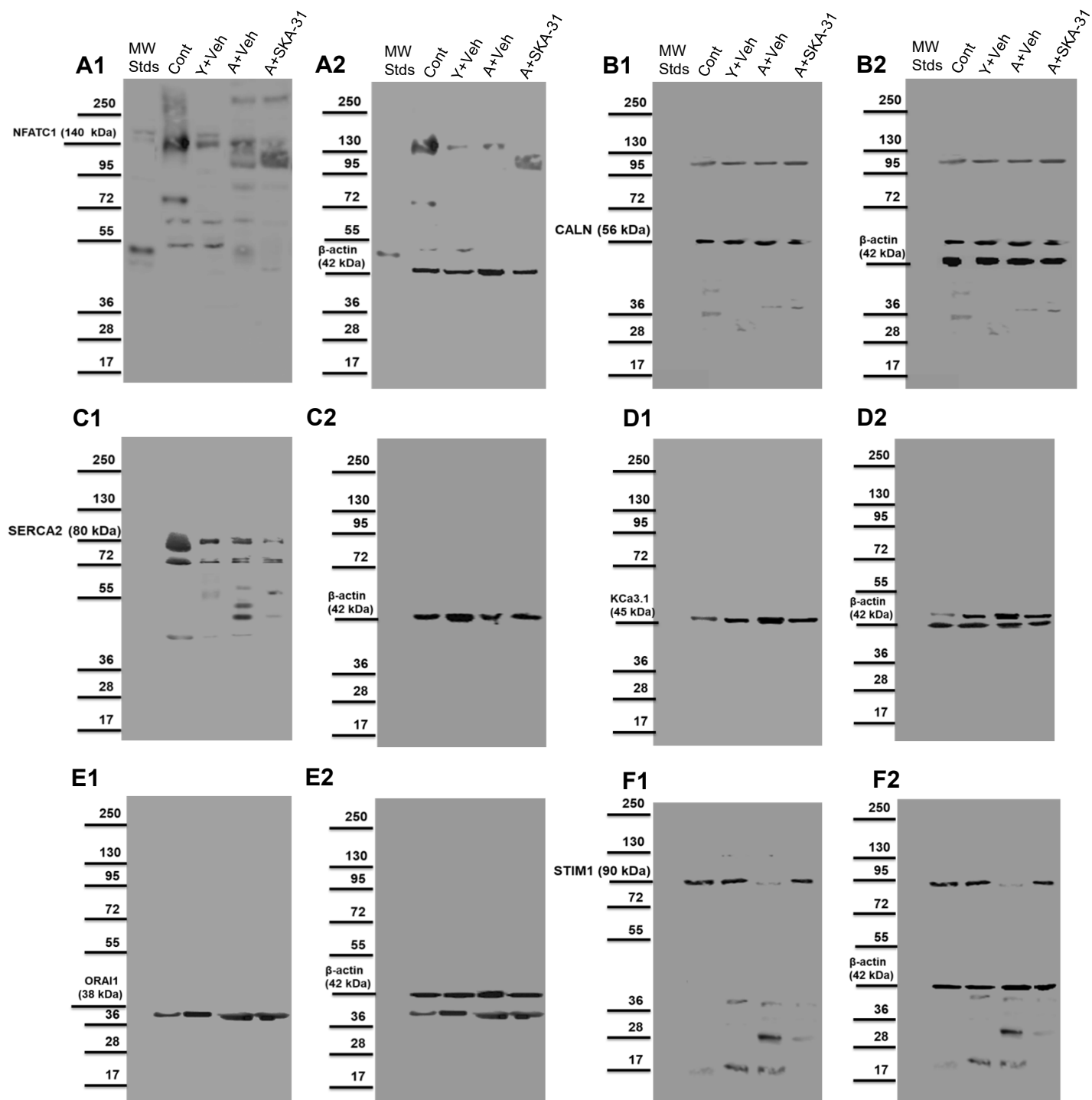
Supplementary Figure 9

Supplementary Figure 10 – Detection of calcineurin/NFAT signalling components in isolated T cells from vehicle and SKA-31 treated young rats. Panels A-H present western blot images and quantification of NFATC1 (A), calcineurin catalytic subunit (B), Kv1.3 channel (C), KCa3.1 channel (D), ORAI1 channel (E), SERCA2 ATPase (F), STIM1 (G) and IP3R1 (H) in splenic CD4⁺ T cells from vehicle (white) and SKA-31-treated young rats (black). For each primary antibody tested, a lysate derived from a tissue or recombinant cell (RC) expressing the target protein was included as a positive control, and is displayed in lane 1 of each blot. Staining intensities of the selected immunoreactive bands detected in T cell homogenates are expressed as a ratio to the expression of β -actin in the same homogenate (n = 4 animals/group; n=3 for IP3R1). No statistical differences in expression were observed between vehicle and SKA-31 treated animals, as determined by an unpaired Student's t-test. Panel I displays the mRNA expression of key signaling proteins in splenic CD4⁺ T cells from vehicle and SKA-31 treated young rats. The qPCR results for each target have been normalized to the mRNA expression of GAPDH, which was calculated using REST software and analysed by an unpaired Student's t-test (n = 3-5 animals/group).



Supplementary Figure 10

Supplementary Figure 11 – Representative full length western blots for target proteins detected in splenic T cells isolated from young and aged rats. T cells were isolated as described in the Methods and Materials from the spleens of young rats treated with vehicle (lane denoted Y+Veh), or aged rats administered either vehicle (A+Veh) or 10 mg/kg SKA-31 (A+SKA-31) for 8 weeks. Tissues or cells with expected positive reactivity for the primary antibody in question were utilized as positive controls (lane denoted Cont) for the immuno-detection process. The electrophoretic positions of molecular weight markers are indicated on the left-hand side of each blot. The identification and electrophoretic position of the target protein of interest for each blot are also indicated on the left-hand side. Blot images are arranged in pairs (e.g. A1 and A2), with the target protein of interest shown in blot #1 and associated β -actin staining on the same membrane displayed in the blot labelled #2. In some cases, the β -actin blot displays protein bands arising from the initial detection of the target protein of interest. This signal carryover occurred mainly from the use of monoclonal antibodies to detect each separate target, and the use of a common HRP-linked secondary antibody. Note that blots were not chemically stripped between the first and second rounds of immuno-detection.



Supplementary Figure 11